

Oridonin inhibits breast cancer cell proliferation and migration through suppressing COX-2 expression

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Abstract

Oridonin, a biologically active substance isolated from the plant *Rabdosia rubescens*, is recently reported to have an inhibitory effect on breast cancer. However, its mechanism is still unclear. According to previous findings, increased COX-2 expression has been associated with a higher incidence of breast cancer. Therefore, this study aims to explore whether oridonin inhibits cell proliferation and migration by suppressing the expression of COX-2 in human breast cancer cells MDA-MB-231 in vitro and in vivo conditions. In this study, human breast cancer cells MDA-MB-231 will be treated with increasing concentrations and for various durations with oridonin to investigate whether oridonin inhibits cell migration, decrease proliferation, and reduce tumors in mice through suppressing the expression of COX-2. A positive control is a taxol. A negative control is medium. Cell migration will be tested by Transwell assay. The proliferation will be detected by MTT assay. The western-blotting assay will be used for COX-2. In nude mice, the in vivo anti-tumor efficacy of oridonin will be examined. The study's result will provide insight into the effect of oridonin on the proliferation and migration of human breast cancer cells MDA-MB-231. It will also provide reference for the mechanism of how oridonin inhibits breast cancer and lay the foundation for future studies on the molecular basis of oridonin's pharmacological activity in the future.

Keywords: oridonin, breast cancer, COX-2, proliferation, migration

1. Introduction

With a high incidence rate and death, breast cancer is one of the most prevalent malignant tumors in women. According to the data of the WHO International Cancer Research Institute, there were 2.26 million new cases of breast cancer in the world in 2020, accounting for 11.7% of new cancer cases, making it the largest cancer in the world [1]. For patients who have been diagnosed with breast cancer, different treatment strategies are used, such as targeted therapy, hormone therapy, radiotherapy, surgery and chemotherapy. Nowadays, chemotherapy continues to be the major method of treatment for breast cancer among them. However, conventional chemotherapy drugs have large side effects and are easy to be resistant to drugs [2] for example, commonly display resistance to chemotherapy, and this is the major obstacle to a better prognosis of patients. Frequently, literature presents studies in monolayer cell cultures, 3D cell cultures or in vivo studies, but rarely the same work compares results of drug resistance in different models. Several of these works are presented in this review and show that usually cells in 3D culture are more resistant to drugs than monolayer cultured cells due to different mechanisms. Searching for new strategies to sensitize different tumors to chemotherapy, many methods have been studied to understand the mechanisms whereby

cancer cells acquire drug resistance. These methods have been strongly advanced along the years and therapies using different drugs have been increasingly proposed to induce cell death in resistant cells of different cancers. Recently, cancer stem cells (CSCs). Therefore, it is urgent to find novel therapeutic methods and strategies. Recently, the use of herbal medicine to treat breast cancer patients is considered to be a natural alternative.

Oridonin, which has the molecular formula $C_{20}H_{28}O_6$, is a biologically active substance isolated from the plant *Rabdosia rubescens* (Hemsl.) Hara. It is a natural organic compound of kaurene type tetracyclic diterpenoid (Figure 1). Numerous pharmacological actions of oridonin have been shown through studies, such as anti-inflammatory, antioxidant, immune modulating, antibacterial, etc[3]. In recent years, it has been established that oridonin also has anti-tumor activity and has obvious inhibitory effect on esophageal cancer[4], prostate cancer[5], gastric cancer[6], lung cancer[7] and other tumor cells. Research has supported the idea that oridonin inhibits breast cancer growth and metastasis and this inhibitive effect is likely to be driven by the inhibition of Notch signaling pathway and the resulting increased apoptosis[8]. However, the exact mechanism how oridonin prevents the development and progression of breast cancer is still unknown.

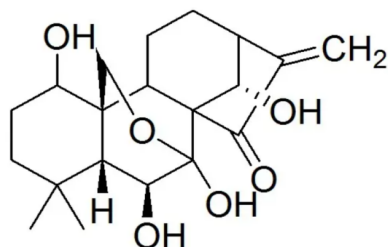


Figure1. Chemical structure of oridonin

COX-2 is a rate limiting enzyme for prostaglandin peroxidase synthesis. It plays a physiological and pathological role by inducing arachidonic acid to transform into prostaglandin. Multiple malignant tumors may be promoted by COX-2 over-expression, which is brought on by the activation of growth factors, inflammatory cytokines, endotoxins, or oncogenes [9]. After down-regulation of COX-2 expression, it can promote cell apoptosis, inhibit angiogenesis, enhance cellular immunity and act as an anti-tumor agent [10] more evidence is needed for clinical application. The purpose of this study was to investigate the feasibility of COX2 inhibition as a strategic treatment modality for head and neck carcinoma (HNC). Previous research has demonstrated a strong correlation between breast cancer incidence and high COX-2 expression [11]. Although the molecular mechanism of how COX-2 triggers and promotes cancer remains to be further studied, COX-2 is thought to be a viable target for tumor therapy due to its significant role in the development of malignancies.

Therefore, this article aims to investigate whether oridonin could inhibit human breast cancer cell migration, decrease proliferation and reduce tumors. Moreover, the effect of oridonin on COX-2 expression in breast cancer cells MDA-MB-231 will be explored.

2. Hypothesis

I predict treatment with increasing concentrations and for various durations with oridonin can inhibit cell migration, decrease proliferation, and reduce tumors in mice through suppressing the expression of COX-2 in human breast cancer cells MDA-MB-231. For *in vitro* experiments, the positive control is taxol and the negative control is culture medium. For *in vivo* experiments, the positive control is taxol and the negative control is saline.

3. Materials and Methods

3.1. Reagents

Oridonin (purity >98%) dissolved in dimethyl sulfoxide (DMSO) as a 100 mM stock solution and stored at -20°C . DMEM, FBS, RPMI lysis, crystal violet staining solution, penicillin, streptomycin.

3.2. Cell line and cell culture

Human breast cancer cell line MDA-MB-231 will be used. These cells will be cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cell Culture will be incubated at 37°C and 5% CO_2 and sub-cultured twice weekly. Cells in good growth condition and in logarithmic phase will be taken for experiment. For *in vitro* experiments, the drug treated group will be treated by oridonin in different concentrations (1, 10, 100, 1000 and 10,000 μM) respectively for 1,6,12 and 24h.

3.3. MTT assay

MTT assay will be applied to detect the effect of oridonin on proliferation of cells. Collect MDA-MB-231 breast cancer cells of logarithmic phase and seed them into a 96 well culture plate at 37°C , 5% CO_2 . After incubated overnight, the cells will be treated by oridonin in different concentrations (1, 10, 100, 1000 and 10,000 μM) respectively for 1,6,12 and 24h. MTT solution will be added to each well and incubated for 4 hours. To dissolve the formazan crystals, remove the medium out of the wells and add DMSO. A microplate reader is used to measure the absorbance of each well. Each treatment will be performed in three separate wells with a control group of cells cultured in a medium containing DMSO. Each experiment will be repeated at least 3 times. The inhibition rate (percentage of control) will be calculated as follows: $[1 - (\text{average absorbance of test sample} / \text{average absorbance of control})] \times 100\%$.

3.4. Transwell migration assay

The migration assay will be carried out by applying a 24-well transwell unit with polycarbonated filters. After treatment by oridonin in different concentrations (1, 10, 100, 1000 and 10,000 μM) respectively for 1,6,12 and 24h, MDA-MB-231 cells will be collected and seeded in upper chamber covered with Matrigel at a density of 4×10^4 cells/well. DMEM plus 10% FBS will be placed in the lower part of the transwell. The cells will be cultured at 37°C with 5% CO_2 for 24 hours to migrate. At room temperature, the migrating cells will be fixed with 4% methanol for 20 minutes and stained with 0.1% crystal violet staining solution. A computerized microscope will be used to capture images. Cell migration will be counted in five separate fields per membrane. Five duplicates of each experiment will be averaged to provide the results.

3.5. Western-blotting assay

Western blotting assay will be used for the detection of COX-2 expression level in human breast cancer cells. After being harvested, RIPA lysis buffer with protease

inhibitors will be used to suspend the cells. Three rounds of five one-second bursts of ultrasonication will be performed on the samples, followed by a 15-minute centrifugation at 13,000 rpm at 4°C. A BCA protein assay kit will be used to measure the amount of protein in the collected supernatants. The proteins will be separated in an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes will first be blocked with TBST (tween 20, 0.05%) containing 5% non-fat milk at room temperature for two hours, followed by an overnight incubation with primary COX-2 antibody at 4°C. The membranes will be treated with secondary antibodies coupled to horseradish peroxidase at room temperature for 2 hours after being washed with TBST. Bands will be detected with the hypersensitive ECL chemiluminescence kit.

3.6. Tumor inhibition assay *in vivo*

The xenogeneic mouse model will be established to further evaluate the effect of oridonin on tumor growth *in vivo*. Thirty male BALB/C athymus nude mice, weighing between 18 and 20 g (4-6 weeks old), will be randomly divided into positive control group (taxol), negative control group (saline) and three oridonin groups, with six mice in each group. Each nude mouse will get a subcutaneous injection of MDA-MB-231 breast cancer cells close to the axillary fossa. When tumor forms and grows to a volume of about 100 mm³, mice in the positive control group, negative control group and oridonin group will be injected with taxol, saline and 2.5, 5 and 10 mg/kg oridonin respectively by intraperitoneal injection. Each group will be treated once a day for 21 days. At the same time, body weight and the tumor sizes will be measured every two days and the tumor sizes will be measured by a caliper. After 21 days, all experimental mice will be killed, tumors will be removed, and their weights will be measured. The experiment will be repeated three times.

3.7. Statistical analysis

Student's t-test will be used in MTT assay, Transwell migration assay, Western-blotting assay and *in vivo* experiment. $P < 0.05$ will be considered to be a statistically significant difference. SPSS software will be used for statistical analysis.

4. Possible Results

In Result 1, cell proliferation is decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 2, cell proliferation is decreased in MTT

assay, cell migration is decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is not decreased in nude mice.

In Result 3, cell proliferation is decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 4, cell proliferation is decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is not decreased in nude mice.

In Result 5, cell proliferation is decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 6, cell proliferation is decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is not decreased in nude mice.

In Result 7, cell proliferation is decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 8, cell proliferation is decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is not decreased in nude mice.

In Result 9, cell proliferation is not decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 10, cell proliferation is not decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is not decreased in nude mice.

In Result 11, cell proliferation is not decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 12, cell proliferation is not decreased in MTT assay, cell migration is decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-

blotting assay and the tumor size is not decreased in nude mice.

In Result 13, cell proliferation is not decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 14, cell proliferation is not decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is decreased in

nude mice.

In Result 15, cell proliferation is not decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is decreased in nude mice.

In Result 16, cell proliferation is not decreased in MTT assay, cell migration is not decreased in Transwell assay, the expression of COX-2 is not down regulated in Western-blotting assay and the tumor size is not decreased in nude mice (Table 1).

Table 1: Possible Results

	Decreased cell proliferation?	Decreased cell migration?	Decreased COX-2 expression?	Decreased xenograft tumor size?	Supporting Hypothesis ?
Result 1	+	+	+	+	Yes
Result 2	+	+	+	-	Partially
Result 3	+	+	-	+	Partially
Result 4	+	+	-	-	Partially
Result 5	+	-	+	+	Partially
Result 6	+	-	+	-	Partially
Result 7	+	-	-	+	Partially
Result 8	+	-	-	-	Partially
Result 9	-	+	+	+	Partially
Result 10	-	+	+	-	Partially
Result 11	-	+	-	+	Partially
Result 12	-	+	-	-	Partially
Result 13	-	-	+	+	Partially
Result 14	-	-	+	-	Partially
Result 15	-	-	-	+	Partially
Result 16	-	-	-	-	No

Note. “+” means the measurement changes similarly to positive control taxol.

“-” means the measurement changes similarly to negative control medium/saline.

5. Discussion

Recently, oridonin has been established to have anti-tumor action and a considerable inhibitory effect on breast cancer. However, its mechanism is still uncertain. Previous studies have shown a significant association between increased COX-2 expression and the development of breast cancer. Therefore, the goal of this study is to investigate whether oridonin inhibits cell proliferation and migration through suppressing the expression of COX-2 in human breast cancer cells MDA-MB-231 both *in vitro* and *in vivo* conditions.

Possible results are shown in Table 1 above to provide an

overview of whether oridonin inhibits cell proliferation and migration via suppressing COX-2 expression in MDA-MB-231 breast cancer cells. Result 1 is totally in line with the hypothesis, which means oridonin inhibits cell migration, decrease proliferation, and reduce tumors in mice through suppressing the expression of COX-2. Results 2-15 partially agree with the hypothesis. Result 16 demonstrates that oridonin has no significant effect on the proliferation and migration of MDA-MB-231 breast cancer cells and does not reduce tumor size in mice.

Result 1 is totally in line with the hypothesis. The proliferation and migration of MDA-MB-231 breast

cancer cells in the oridonin treated group are inhibited in a way similar to positive control taxol. This result is consistent with another study which looked into the impact of oridonin on EMT and angiogenesis in breast cancer [12]. Additionally, the expression of COX-2 drops considerably in the breast cancer cells exposed to oridonin in a way similar to positive control taxol, demonstrating that the downregulation of COX-2 expression may contribute to how oridonin inhibits breast cancer. It was mentioned in another paper that andrographolide might inhibit breast cancer by reducing COX-2 expression and angiogenesis through inactivation of p300 signaling and VEGF pathway[13]. Given this information, it makes sense to further explore the mechanism of oridonin with reference to this direction. As for the tumor inhibition assay *in vivo*, the possible result reflects that oridonin has an anti-tumor effect on the growth of xenografted MDA-MB-231 cells in nude mice. This outcome is similar with a prior research which applied oridonin to prevent breast cancer by blocking Notch signaling, where the anti-tumor effect of oridonin in nude mice with xenografted 4T1 cells was demonstrated by the decrease in tumor volume and weight [8].

Result 2 partially supports the hypothesis. In Result 2, oridonin inhibits cell migration, decreases proliferation and down-regulates COX-2 expression *in vitro* while fails to reduce tumors in nude mice *in vivo*. Several reasons may lead to this result. For example, oridonin is decomposed by enzymes within the nude mice or oridonin is eliminated without entering the tumor. In the future experiments, the expression of COX-2 can be further detected by the chromatin immunoprecipitation assay. Result 3 and Result 4 partially support the hypothesis. In Result 3, oridonin does not down-regulate COX-2 expression but inhibits cell migration, decreases proliferation and reduces tumors in mice in a way similar to positive control taxol. In Result 4, oridonin does not decrease the expression of COX-2 and the tumor size. These findings may suggest that, despite oridonin's inhibitory effect on the migration and proliferation of breast cancer cells, the production of COX-2 may not directly be responsible for this effect. In order to investigate the mechanism of oridonin inhibiting breast cancer, we can draw lessons from the known potential mechanism of oridonin inhibiting other malignancies. For example, previous research has showed that oridonin, as an AKT inhibitor, can slow the growth of esophageal squamous cell carcinoma (ESCC) by weakening the AKT signal [4].

Result 5 partially supports the hypothesis. In Result 5, oridonin decreases cell proliferation, down regulates COX-2 expression and reduces xenograft mouse tumor in a way similar to positive control taxol but does not inhibit

cell migration. This result indicates that inhibition of tumor *in vivo* may not be directly related to cell migration. Result 6,7 and 8 partially supports the hypothesis. In these possible results, oridonin shows inhibitory effect on cell proliferation but does not affect cell migration significantly. Future experiments such as wound healing assay are needed to further detect cell migration ability. Additionally, flow cytometry analysis is required to confirm the validity of the MTT assay.

Result 9 partially supports the hypothesis. In Result 9, oridonin does not decrease proliferation but inhibits cell migration, suppresses COX-2 expression and reduces tumors in nude mice in a way similar to positive control taxol. This result may be related to the administration concentration of oridonin. In a study on oridonin inhibiting the migration of small cell lung cancer cells, it was mentioned that low concentrations of oridonin had little impact on cell viability, while high concentrations of oridonin significantly inhibited cell viability[7]. Future experiments can be carried out by adjusting the concentration range of oridonin. Result 11 partially supports the hypothesis. In Result 11, oridonin decreases cell migration and xenograft tumor size in the way similar to positive control taxol. This phenomenon might indicate a close connection between cell migration and tumor development. Result 10,12 partially supports the hypothesis. In these possible results, oridonin shows inhibitory effect on cell migration but does not affect cell proliferation and tumor size significantly. Future experiments such as wound healing assay and CCK-8 assay are needed to further detect cell migration ability and viability.

Result 13 and 15 partially supports the hypothesis. In Result 13 and 15, oridonin does not inhibit cell proliferation and migration but help reduce the xenograft tumor size in mice. These results demonstrate that although oridonin does not inhibit cell migration and proliferation *in vitro*, it inhibits tumor with the help of other active substances *in vivo*. Result 14 partially supports the hypothesis. In Result 14, oridonin does not decrease cell proliferation, migration and tumor size in mice but up-regulates the expression of COX-2 in a way similar to the positive control taxol. This result means that although oridonin affects the expression of COX-2, it has no significant inhibitory effect on cancer cells and tumor development *in vivo* and *in vitro*. Future experiments such as wound healing assay, CCK-8 assay, flow cytometry analysis and *in vivo* experiments are needed for further determination.

Result 16 is completely contradictory to the hypothesis. The measurement results of cell proliferation, cell migration, COX-2 expression and tumor size all change similarly to negative control culture medium/

saline, indicating that oridonin has no significant effect on the proliferation and migration of MDA-MB-231 breast cancer cells and does not reduce tumor size in mice. Future experiments such as wound healing assay, CCK-8 assay, flow cytometry analysis, chromatin immunoprecipitation and *in vivo* experiments are needed for further determination in oridonin and other potential drugs.

It should be emphasized that the molecular phenotype of the tumor may be connected to how oridonin affects the proliferation and migration of breast cancer cells. In this experiment, human breast cancer cell line MDA-MB-231 is applied, which does not mean that the experimental results are necessarily applicable to other cells. Furthermore, animal experiments have shown that high concentrations of oridonin have obvious cytotoxicity on hepatic tissue and myocardial tissue[14], which means the dosage of oridonin should be carefully selected in clinical use. In addition, the expression of COX-2 may be decreased due to the decline of cell viability and apoptosis. Therefore, future experiments such as the chromatin immunity assay are required to confirm whether the inhibition mechanism of oridonin is directly related to the expression of COX-2.

6. Discussion on concentration and treatment duration

If the measurement results of the four experiments are concentration and treatment duration dependent, it can be concluded that oridonin really plays a role in inhibiting cell proliferation, decreasing migration and down regulating the expression of COX-2 in human breast cancer cells MDA-MB-231. However, concentration independence means that oridonin does not specifically affect the result and it is possible that other contaminants in the buffer killed the cancer cells instead.

7. Conclusion

In conclusion, this study aims to explore whether oridonin inhibits cell proliferation and migration through suppressing the expression of COX-2 in human breast cancer cells MDA-MB-231. The inhibitory effects of oridonin on breast cancer will be determined both *in vitro* and *in vivo* conditions. However, the precise molecular mechanism behind these effects has not yet been fully explained. Therefore, future experiments are required to continue exploring the molecular basis of its pharmacological activity.

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